ADP–RIBONOLACTONE: A POTENTIAL ACTIVATED INTERMEDIATE ANALOGUE OF NAD-GLYCOHYDROLASE

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Received 18 November 1976

1. Introduction

Aldono-1,5-lactones are efficient competitive inhibitors with high specificity for glycosidases that catalyze the hydrolysis of glycosides derived from the same aldoses [1]. The inhibition has been attributed to a conformational (half-chair) and electronic (sp^2 hybridization and electron delocalization) similarity between the lactone and the hypothetical glycosyl-ion which is involved as an intermediate in substrate transformation [2]. A rationale was provided by application of absolute reaction rates theory to enzymatic catalysis. It predicts that an enzyme develops an enhanced affinity for the transition state of a catalyzed reaction, and therefore for 'transition state analogues', comparatively to its substrates [3]. A classical example for glycosidases is that of lysozyme where the glycosyl group at which cleavage occurs must be distorted, according to X-ray analysis, into a half-chair conformation in order to fit into the active site. This substrate distortion towards the structure of the transition state is believed to be a major factor responsible for the mode of action of that enzyme [4]. A 1,5-lactone derived of chitotetraose, an analogue for the proposed transition-state, was found to a potent ligand for lysozyme [5].

NAD-glycohydrolases (EC 3.2.2.6) catalyze the hydrolysis of NAD at the nicotinamide–ribose linkage. The kinetic mechanism of calf spleen NAD glycohydrolase was found to be consistent with an Ordered Uni Bi, the formation of an ADP-ribosyl intermediary complex (E–ADP-rib) being rate determining [6]. The reactivity of E–ADP-rib versus nucleophiles was in favor of an oxocarbonium ion intermediate in the NAD transformation [6,7]. In attempting to assess this point further we have prepared ADP-ribonolactone. This compound by its ribono-1,4-lactone moiety should be an analogue of the postulated ribosyl ion intermediate. This latter being a high energy intermediate, according to the Hammond postulate [8], the lactone should resemble the structure of the transition state more closely than the reaction product ADP-ribose. One would therefore predict that ADP-ribonolactone is bound more tightly by the NAD-glycohydrolase than ADP-ribose, which was previously found to be a competitive inhibitor of NAD hydrolysis [6].

The present report describes the preparation of ADP-ribonolactone and its properties as an inhibitor of calf spleen NAD-glycohydrolase.

2. Materials and methods

2.1. *Chemicals*

β-NAD, D-ribose-5-phosphate, D-ribonic acid-γ-lactone (ribonolactone) ADP-ribose and AMP were purchased from Sigma Chemical Co. Bio-Gel P-4 (100–200 mesh) was obtained from Bio-Rad Laboratories. 2,3,5-Triphenyltetrazolium chloride and all other chemicals were Merck products.

2.2. *Enzyme*

Calf spleen NAD glycohydrolase was solubilized and partially purified according to the procedure previously published [9]. The specific activity was about 2.4 units/mg of protein.

2.3. *Preparation of ADP-ribonolactone*

Oxidation of ADP-ribose by alkaline iodine,

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followed by an ion exchange step, yielded a mixture of ADP-ribonic acid and ADP-ribonolactone.

ADP-ribose (0.177 mmol) in 40 ml water was oxidized to ADP-ribonic acid with iodine (0.01 M) in presence of NaI (0.05 M), Li_2CO_3 (0.05 M), at 4°C in the dark [5]. Aliquots of the reaction mixture were acidified with sulfuric acid (5 N) and the residual iodine titrated with sodium thiosulfate. The reaction was essentially complete after 2 h and the reduction of one equivalent of I_2. The reaction mixture was then acidified to pH 3.5 with sulfuric acid (5 N), and excess iodine extracted with benzene (4 × 50 ml). After lyophilization of the aqueous solution, the iodide salts were extracted with cold acetone (−20°C). The residue was redissolved in water (5 ml) and chromatographed (ascending) on a column (2.8 × 70 cm) of Bio-Gel P-4 with water as eluant. The salt of ADP-ribonic acid was thus separated from AMP and remaining salts.

Oxidized ADP-ribose was transferred onto a column (1.8 × 10 cm) of Dowex 50 X 2 (H^+ form) equilibrated with water. After elution (water) the fractions containing free ADP-ribonic acid were pooled and immediately lyophilized. The obtained powder was redissolved in water and rechromatographed on the Bio-Gel column in order to eliminate AMP which was formed during the ion exchange step. The combined fractions containing the ADP-ribosyl derivatives were lyophilized and the white powder kept under vacuum in a dessicator over P_2O_5 for several days. The product obtained (average yield 30%) was analyzed (vide infra) and consists of a mixture of ADP-ribonolactone and ADP-ribonic acid, free of other contaminants (electrophoresis).

2.4. *Characterization of ADP-ribonolactone*

The presence of ADP-ribonolactone was determined in the final preparation according to the following criteria.

(i) A reducing sugar test using 2,3,5,-triphenyltetrazolium chloride [10], and ribose-5-phosphate as standard, was completely negative. This confirms that the oxidation of ADP-ribose occurred at the anomeric carbon.

(ii) The ultraviolet absorption spectra, using a Cary 118 spectrophotometer, was characteristic of an unmodified adenosine system (λ_{max} 259 nm, ϵ = 15.400 M^{-1} cm^{-1}, pH 7) and indicates that the C-8 of the adenine ring remains unsubstituted under our experimental conditions [11,12].

(iii) ADP-ribonolactone gave a positive hydroxylamine–ferric chloride test [13]. Using ribonolactone as standard, this method, in addition to absorbance measurements, allowed us to determine the percentage of ADP-ribonolactone present in the mixture. An excellent agreement was reached when the lactone was titrated with sodium hydroxide (pH-stat).

(iv) Base-catalyzed hydrolysis of ADP-ribonolactone was studied between pH 8.0 and 9.0, at 37°C and I = 0.1 M. The hydrolysis is unimolecular in hydroxide and a second-order rate constant of 3.3×10^3 $min^{-1}M^{-1}$ was calculated (using pK_W = 13.68 at 37°C). Under the same conditions $k_{OH^-} = 4.0 \times 10^4$ $min^{-1}M^{-1}$ for ribonolactone.

(v) Electrophoresis were performed at 3 000 V in 0.05 M sodium acetate buffer (pH 5.0) on Schleicher and Schüll paper (No. 2043). The ADP-ribosyl derivatives, obtained by oxidation of ADP-ribose, revealed two spots under ultraviolet; ADP-ribonic acid was identified after alkaline hydrolysis of the lactone ring. The following mobilities (10^{-5} cm^2V^{-1} sec^{-1}) were measured (towards the anode): NAD (3.8), AMP (6.7), ADP-ribonolactone (9.8) ADP-ribose (10.7) and ADP-ribonic acid (11.9).

3. Results

The anomeric carbon of ADP-ribose was oxidized with alkaline iodine and the resultant ADP-ribonic acid could be converted partially into ADP-ribonolactone after a cation-exchange step. The mixture obtained generally contained 30–50% in lactone. The criteria described under Materials and methods make us confident of the characterization of ADP-ribonolactone. Under conditions of prolonged acid treatment a higher proportion of lactone could be obtained. However in this case the yields were poor, owing to a very fast decomposition of ADP-ribonic acid. The markedly increased sensitivity of the pyrophosphate bond of ADP-ribonic acid compared to ADP-ribose, under acidic conditions, can be tentatively explained on the following basis: the acid catalyzed 1,4-lactonization of the ribonic-acid moiety is in competition with the highly favorable formation of a reactive five-membered cyclic phosphate and AMP

(i.e., the 4′-hydroxyl reacting with the pyrophosphate bond). Similar examples are well known in phosphate ester chemistry [14].

Alkaline iodine oxidation of the ribose moiety is a reaction of general interest which was used in our laboratory in order to distinguish ADP-ribose from C-1′ substituted ADP-ribosides, e.g., titration of consumed iodine gave the percent of ADP-ribose present in a mixture of ADP-ribose and methyl-ADP-riboside [7]. In this study we found that ADP-ribonic acid is strongly absorbed by a DEAE-cellulose column and cannot be readily purified by ion-exchange chromatography; however this property allowed us to prepare, after the oxidation step, pure β-methyl-ADP-riboside from a mixture of ADP-ribose and methyl-ADP-riboside [15].

The alkaline hydrolysis of ADP-ribonolactone is relatively fast, e.g., half-life 30 min at pH 8.51 and 37°C. However from the second-order rate constant one can predict that, under our experimental conditions, no lactone ring opening should occur during the period of initial rate measurements of the enzymic hydrolysis of NAD in the presence of ADP-ribonolactone. This point was confirmed by control experiments using the neutral hydroxylamine–ferric chloride test.

3.1. *Inhibition properties of ADP-ribonolactone*

NAD hydrolysis catalyzed by calf spleen NAD glycohydrolase was inhibited competitively by a mixture of ADP-ribonolactone and ADP-ribonic acid. The double reciprocal plot is shown in fig.1.

ADP-ribonic acid obtained by alkaline hydrolysis of ADP-ribonolactone behaved also as a competitive inhibitor (fig.2).

The value of apparent enzyme-inhibitor dissociation constants of ADP-ribose (plot not shown) and its analogues are listed in table 1. Competitive inhibition of NAD-glycohydrolase in the presence of two competitive inhibitors, ADP-ribonolactone and ADP-ribonic acid (fig.1), can be analyzed according to the multiple inhibition kinetics method as described by Yagi and Ozawa [15]. A plot of V_o/V_i against $(i_1 + i_2)$ i.e., the total concentration of the two inhibitors, should be linear when both inhibitors interact with the same site of the enzyme and are mutually exclusive (interaction constant [17]: $\alpha = \infty$). This was found to be the case for the pair of

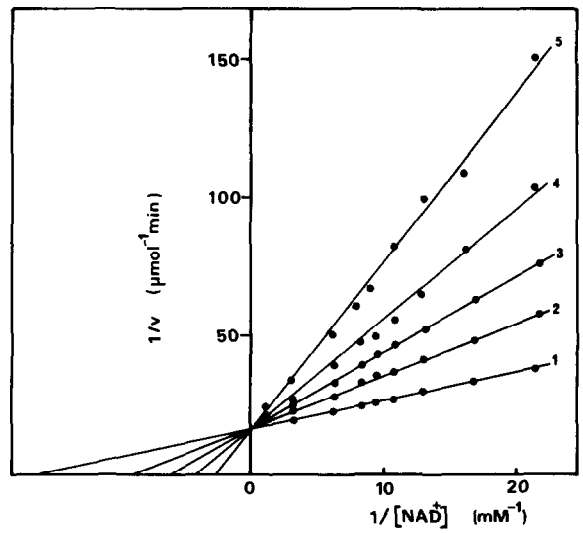

Fig.1. Multiple inhibition of NAD-glycohydrolase by ADP-ribonolactone and ADP-ribonic acid (30 : 70). Initial rates of NAD hydrolysis catalyzed by calf spleen NAD-glycohydrolase (0.06 unit) were measured by a pH-stat method [9] at pH 5.8, I = 0.1 M and 37°C, in a total volume of 1.9 ml. The total concentrations (mM) of ADP-ribosyl derivatives used were as follows: 1, none; 2, 0.125; 3, 0.235; 4, 0.39; 5, 0.785.

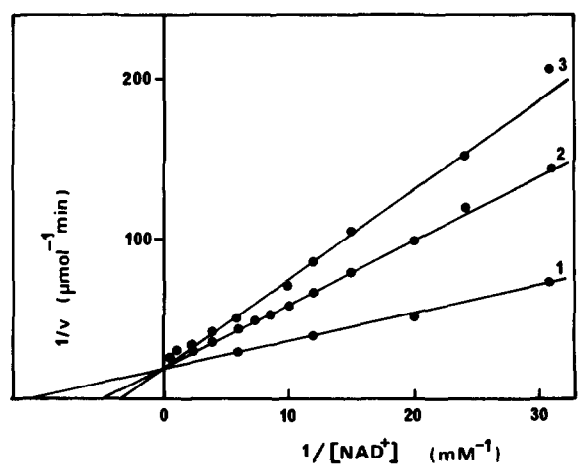

Fig.2. Inhibition of NAD hydrolysis by ADP ribonic acid. Fig.1 provides the experimental conditions. ADP-ribonic acid concentrations (mM) used: 1, none; 2, 0.25; 3, 0.37.

Table 1
Inhibition of NAD-glycohydrolase by ADP-ribose analogues

Inhibitor	K_i
ADP-ribose	1.0×10^{-3} M
ADP-ribonolactone + ADP-ribonic acid (3:7)	1.65×10^{-4} M
ADP-ribonic acid	2.0×10^{-4} M
ADP-ribonolactone	1.15×10^{-4} M[a]

[a] Extrapolated value (see text)

K_i values were calculated from Lineweaver-Burk plots (fig.1,2). Under these experimental conditions K_m (NAD) = 62 μM.

inhibitors studied (fig.3). Knowing the concentrations i_1 and i_2, and one inhibitor dissociation constant, e.g., K_{i1}, one can calculate K_{i2} [17]. These values are related by the following expression (when $\alpha = \infty$):

$$\frac{i_1 + i_2}{K_{i1,2}} = \frac{i_1}{K_{i1}} + \frac{i_2}{K_{i2}}$$

where $K_{i1,2}$ is the apparent K_i obtained in the presence of I_1 and I_2. Application of this method to the present study gave for ADP-ribonolactone a $K_i = 1.15 \times 10^{-4}$ M.

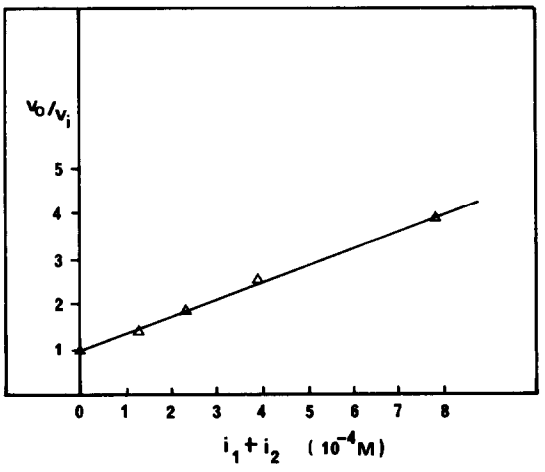

Fig.3. Yagi-Ozawa plot for multiple inhibition of NAD glycohydrolase by ADP-ribonolactone and ADP-ribonic acid. The data for this plot are taken from fig.1.

4. Discussion

This study indicates that ADP-ribonolactone has a 9 times higher affinity for the active site of calf spleen NAD-glycohydrolase relative to ADP-ribose. This result suggests that ADP-ribonolactone by its 1,4-ribonolactone moiety bears some resemblance to ADP-rib, the high energy intermediate occurring after the enzymic induced nicotinamide–ribose bond breaking. The 1,4-ribonolactone has a rigid structure where C_3 points out of a plane (probably C_3-endo) delineated by the atoms $C_2-C_1-O-C_4$ [18]. The carbonyl at C_1 has a sp^2 hybridization and the electrons may be delocalized, C_1-O having a double bond character (valence-bond resonance). Such a structure is closely related to the postulated intermediary ADP-ribosyl oxocarbonium ion where the positive charge at the anomeric carbon C_1 is also delocalized along the C_1-O bond.

In contrast to the 1,4-ribonolactone, ribofuranoses have a high flexibility and their conformation alternates via pseudo-rotation between envelope and twist forms [19,20]. For nucleotides and NAD in solution, the ribose ring adopts a two state equilibrium C_3-endo ⇆ C_2-endo, with a preference for the C_2-endo conformation [21,22]. When NAD is bound to an enzyme X-ray structural analysis have indicated, on best fit bases, a preference for C_2-endo and C_3-endo respectively for cytoplasmic malate dehydrogenase and M-4 lactate dehydrogenase. However the energy differences between these two conformations are quite small [22]. In comparison to ADP-ribonolactone and the postulated ADP-ribosyl oxocarbonium ion, the ribofuranose moiety of ADP-ribose lacks that conformational rigidity and its C_1 has a sp^3 hybridization. However the energy barrier to adopt a related C_3-endo conformation is low. It follows that the differences between 1,4-lactone and ribose in terms of 'high energy intermediate analogues' are less straightforward that in the case of hexoses where the change between a chair and half-chair is more important. The strained 1,5-aldonolactone half-chair has also a much higher energy than the corresponding aldose chair conformation. Such a difference for the two systems is illustrated by the alkaline hydrolysis rate of 1,5-lactones and 1,4-lactones, the latter being more stable [23]. The existence of a subtle change between the ribose and ribonolactone moities (i.e.,

electronic rather than conformational) might be reflected in the relatively small difference in binding energy ($\Delta\Delta G$ = 1.3 kcal mol^{-1}) between the enzyme, ADP-ribose and ADP-ribonolactone (the high energy intermediate analogue). No other example has been described to our knowledge for a nucleosidase. In glycosidases where similar intermediary glycosyl oxocarbonium ions have been proposed, the difference in binding energies between the aldose and the corresponding 1,5 aldonolactone are comprised to be between one and two orders of magnitude [3]. For example lysozyme binds the 1,5-lactone derived from tetra-acetylchitotetraose 32 times better than the unmodified saccharide [5]. Modest differences were observed, between 1,5-D-gluconolactone and D-glucose interaction energies with sucrase although an oxocarbonium ion intermediate is probable [24].

In contrast to glycosidases where the aldonic acids are noninhibitory, ADP-ribonic acid has a good affinity for the active site of calf spleen NAD glycohydrolase. Because of its open-chain structure, this compound cannot be related to ADP-ribose and ADP-ribonolactone. It is possible that owing to its conformational flexibility a residue of this ribonic acid can interact with a side chain which is not normally involved in the binding of the product by the enzyme.

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